Growth suppression by *p18*, a p16$^{INK4/MTS1}$- and p14$^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function

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The D-type cyclin-dependent kinases CDK4 and CDK6 are complexed with many small cellular proteins (p14, p15, p16, p18, and p20). We have isolated cDNA sequences corresponding to the *MTS2* genomic fragment that encodes the CDK4- and CDK6-associated p14 protein. By use of a yeast interaction screen to search for CDK6-interacting proteins, we have also identified an 18-kD human protein, p18, that is a homolog of the cyclin D–CDK4 inhibitors p16 (*INK4A/MTS1*) and p14 (*MTS2/INK4B*). Both in vivo and in vitro, p18 interacts strongly with CDK6, weakly with CDK4, and exhibits no detectable interaction with the other known CDKs. Recombinant p18 inhibits the kinase activity of cyclin D–CDK6. Distinct from the p21/p27 family of CDK inhibitors that form ternary complexes with cyclin–CDKs, only binary complexes of p14, p16, and p18 were found in association with CDK4 and/or CDK6. Ectopic expression of p18 or p16 suppresses cell growth with a correlated dependence on endogenous wild-type pRb.

[*Key Words*: Cyclin-dependent kinase inhibitors; cell cycle; CDK4 and CDK6 interacting proteins]

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Progression of eukaryotic cells through the cell cycle is regulated by the sequential formation, activation, and subsequent inactivation of a series of structurally related serine/threonine protein kinases. These enzymes consist of a catalytic subunit, a cyclin-dependent kinase (CDK), and a regulatory subunit, a cyclin (Draetta 1990; Sherr 1993). In unicellular yeast, a single prototypic CDK gene, *cdc2* in the fission yeast *Schizosaccharomyces pombe* or *CDC28* in the budding yeast *Saccharomyces cerevisiae*, controls both the G_1/S(Start) and G_2/M transitions in conjunction with the various types of cyclins. In humans and other higher eukaryotes, however, CDKs constitute a multigene family. In general, each of these CDKs can form binary complexes with several different cyclins, and vice versa, although often with a predominant association between a particular CDK and cyclin (for review, see Sherr 1993).

The enzymatic activity of a CDK is regulated at three different levels: cyclin binding and activation, subunit phosphorylation, and association with and inhibition by a group of heterologous small regulatory proteins. In mammalian cells, the number of small cell-cycle regulatory proteins identified by virtue of their ability to interact physically with cyclin or CDK proteins is rapidly increasing. A 21-kD protein, p21, first identified in anti-cyclin D1 immunoprecipitates of human diploid fibroblasts as a component of quaternary cyclin-D complexes that also contain CDKs and proliferating cell nuclear antigen (PCNA; Xiong et al., 1992), was recently identified as a potent inhibitor of all known cyclin–CDK enzymes (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993a) and as a target of p53 transcriptional activation (El-Deiry et al. 1993; Xiong et al. 1993a; Dulic et al. 1994). A p21-related CDK inhibitor, p27^{Kip1}, was found to bind to and inhibit the activity of various cyclin–CDK enzymes and was suggested to function as a potential mediator of G_1 cell-cycle arrest caused by TGF-β treatment or cell–cell contact inhibition (Firpo et al. 1994; Meyerson and Harlow 1994; Polyak et al. 1994a; Slingerland et al. 1994; Toyoshima and Hunter 1994). On further examination of cyclin–CDK complexes in normal and virally transformed cells, two more small cyclin–CDK-associated proteins were identified: p16, a protein that is stimulated to specifically complex with CDK4 in cells lacking pRB function, and p19, a protein present in cyclin-A complexes (Xiong et al. 1993b). The molecular identity of p19 is still under investigation. *p16* was subsequently isolated by yeast two-hybrid screening and was shown to

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form specific binary complexes with CDK4 and CDK6 and inhibit the kinase activity of D-type cyclin-dependent CDK4 (p16^{INK4}; Serrano et al. 1993). *p16* was recently found to be homozygously deleted at a high frequency in a wide variety of human tumor-derived cell lines (*MTS1/CDK4I*; Kamb et al. 1994a; Nobori et al. 1994) and is also mutated or deleted in several specific types of primary tumors (Caldas et al. 1994; Hussussian et al. 1994; Kamb et al. 1994b; Mori et al. 1994; Spruck et al. 1994). The extent to which *p16* is involved in tumorigenesis is currently under intensive investigation.

The major, if not the only, targets of the inhibitory activity of p16 are CDK4 and CDK6 (Serrano et al. 1993; Xiong et al. 1993b). Their preferential association with D-type cyclins, their substrate preference, and the timing of the onset of their kinase activity strongly implicate both CDK4 and CDK6 as physiological kinases for the retinoblastoma susceptibility gene product pRB (Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993; Matsushime et al. 1994; Meyerson and Harlow 1994), whose growth-inhibitory activities are known to be down-regulated by cell cycle-dependent phosphorylation (for review, see Ewen 1994). Elucidation of the mechanism by which the activities of CDK4 and CDK6 are regulated should facilitate our understanding of both cell-cycle control and tumor suppression. Toward this goal, we have further characterized cellular proteins that interact with CDK4 and CDK6.

Results

CDK4 and CDK6 associate with many small cellular proteins

By use of the ^{35}S-metabolic labeling–immunoprecipitation ([^{35}S]-IP] technique, coupled with antigen competition, we have previously identified, mainly in human fibroblasts, a number of cellular proteins that are specifically associated with cyclins, CDKs, and their complexes. These include PCNA, the p53-activated cyclin–CDK inhibitor p21, the CDK4-specific inhibitor p16, and an as-yet-uncharacterized cyclin A-associated polypeptide, p19 (Xiong et al. 1992, 1993b). As many cyclin and CDK genes, particularly D-type cyclins, are expressed in a tissue- and cell type-specific manner (e.g., Inaba et al. 1992; Meyerson et al. 1992; Won et al. 1992; Tam et al. 1994), we reasoned that additional cyclin- or CDK-associated proteins may exist and function in other tissues or cell types. Following this reasoning, the [^{35}S]-IP technique was then employed to search for proteins that associate with CDK4 and its closest relative, CDK6, which shares 71% sequence identity.

[^{35}S]Methionine-labeled lysates were prepared from a wide variety of human cell lines derived from different tissues and immunoprecipitated with antibodies specific to CDK4 and CDK6. In addition to the previously identified p21 and p16, this study revealed at least four other small cellular proteins that appear to associate with CDK4 and/or CDK6 in a specific manner. They include p14 and p15 (present in a cell line of spontaneously immortalized human keratinocytes), HaCaT (Fig. 1A; Boukamp et al. 1988), and p20 and p18 (present in an acute lymphoblastic leukemia cell line, CEM [Fig. 1B]). Each of these proteins represents a distinct polypeptide, as determined by their different gel mobilities, partial V8 proteolysis patterns, and antigenicities (see below). The presence of each of these proteins in anti-CDK4 and/or anti-CDK6 immunocomplexes was effectively competed by the preincubation of each antibody with a competing antigen peptide (Fig. 1A, lanes 2,4; Fig. 1B, lane 6), suggesting their specific associations. Distinct from p16, which exhibits a similar affinity for both CDK4 and CDK6, p18 and p20 appear to preferentially associate with CDK6 (Fig. 1B, lane 5). These results indicate that a potentially large number of small cyclin- and/or CDK-associated proteins are present in cells that have not yet been identified. Experiments described below report the isolation and characterization of cDNAs encoding for two of these proteins, p14 and p18. To avoid adding further confusion to the nomenclature for the CDK inhibitors, we tentatively refer to these genes by the size of their encoded proteins.

MTS2 encodes a functional gene corresponding to p14

A genomic fragment located 10 kb upstream the *p16* locus that contains 93% DNA sequence identity to exon 2 of the *p16* gene (*MTS2*) was identified previously in a search for candidate tumor suppressor genes involved in melanoma (Kamb et al. 1994a). It was not clear, however, whether this genomic fragment corresponded to a pseudogene of *p16* or encoded a functional gene distinct from *p16*. Oligonucleotide primers specific to *MTS2* were used to amplify cDNA templates prepared from a human HeLa cDNA library. A specific DNA fragment was amplified and used as probe to screen the same HeLa library for full-length *MTS2* cDNA clones. Of five λ cDNA clones isolated, the longest one, H2, was analyzed further by DNA sequencing (Fig. 2A). Comparison with the previously reported *MTS2* genomic sequence (Kamb et al. 1994a) indicates that cDNA clone H2 corresponds to *MTS2*. Conceptual translation of this clone revealed a 138-amino-acid open reading frame starting from nucleotide 322. There are neither methionine nor in-frame stop codons 5′ of the putative initiating methionine. The predicted molecular mass of this protein is 14613 daltons (14.6 kD, p14) and it contains 82% protein sequence identity to p16^{INK4} in the aligned region (Fig. 2C; Serrano et al. 1993). The sequence similarity between *MTS2* and *p16* is higher in exon 2 than in exon 1. In vitro-translated MTS2 comigrates with the CDK4- and CDK6-associated p14 seen in HaCat cells (Fig. 1A) and, like p14, also cross-reacts with anti-p16 antibody (data not shown). These results demonstrate that *MTS2* encodes a functional gene that corresponds to the CDK4- and CDK6-associated p14. *p14/MTS2* has a DNA sequence that is almost identical to p15^{INK4B}, which was reported by Hannon and Beach (1994) after the initial submission of this paper. There are, however, quite a few differences between the two sequences in both the untranslated region (Fig.

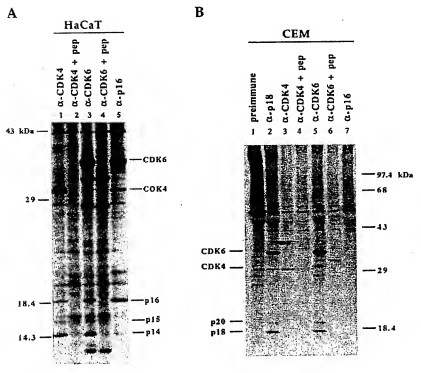


Figure 1. CDK4 and CDK6 associate with many small cellular proteins. [^{35}S]Methionine-labeled lysates were prepared from a cell line of spontaneously immortalized human keratinocytes, HaCaT (*A*), and an acute lymphoblastic leukemia cell line, CEM (*B*). Lysates were immunoprecipitated with indicated antibody with or without preincubation with a competing antigen peptide as indicated at the *top* of each lane. The immunoprecipitated polypeptides were analyzed in each case by SDS-PAGE. The mobility of protein molecular weight standards (GIBCO BRL) and relevant proteins are indicated.

2A, nucleotide positions 10, 52, 146, and 173) and in the coding sequence (Fig. 2A, nucleotide positions 379–387 and 417–423). Differences at nucleotide positions 379–387 (AGCGCCGCG in *p14* and ACGCCG in *INK4B*) result in a change in the amino acid sequence from Ser-Ala-Ala in p14 and to Thr-Pro in INK4B, and differences at nucleotide positions 417–423 result in changes of the amino acid sequence from Asn-Leu-Leu in p14 to His-Ser-Trp in INK4B. We do not know whether these differences are derived from there being two distinct genes, from a cloning or sequencing artifact, or whether the discrepancy might have arisen from gene mutations. In these discrepant areas, the p14 cDNA sequence as presented in Figure 2A is identical to the genomic sequence determined from a clone isolated from a human placenta genomic library (Y. Xiong, unpubl.). p15^{INK4B} is stimulated by TGF-β treatment, suggesting that *p14*/p15^{INK4B} may function as potential effector of TGF-β-induced cell-cycle arrest (Hannon and Beach 1994).

Cloning of p18

To identify other proteins that interact with CDK6, we employed the yeast two-hybrid screening system originally described by Fields and his colleagues (Bartel et al. 1993). The entire open reading frame of human *CDK6* (Meyerson et al. 1992) was fused to the Gal4 DNA-binding domain. This vector directs the expression of a fusion between the DNA-binding domain (amino acids 1–147) of Gal4 and the entire CDK6 protein and was cotransformed into yeast cells with a human HeLa cDNA library constructed in the vector pGADGH (see Materials and methods). Of an estimated 5× 10^6 transformants screened, 42 colonies were His$^+$ and positive for β-galactosidase staining. Plasmid DNA was recovered from positive colonies and analyzed by sequencing. The majority of the 42 clones corresponded to the previously reported p16^{INK4} (Serrano et al. 1993). One clone, 6H10, was found to contain sequences that are distantly related to p16^{INK4} and *MTS2*. The cDNA insert from this clone was used as probe to screen a human HeLa cDNA library to obtain full-length sequences.

One of the longest λ clones, H18, was sequenced and found to contain an apparent full-length coding region, as there is an in-frame stop codon located 6 bp upstream of the putative ATG initiation codon (Fig. 2B). Conceptual translation of this clone revealed an open reading frame with 168 amino acid residues starting at nucleotide 94. The predicted molecular mass of this protein is 18116 daltons (18 ka, p18), and it contains 38% protein sequence identity to p16 (INK4/MTS1) over a 150-amino-acid region and 42% identity to p14 (MTS2/INK4B) over a 129-amino-acid region (Fig. 2C). The sequence similarities between p18 and p16 and between p18 and p14 are higher across the amino-terminal two-thirds of the proteins than in the carboxy-terminal regions.

Further analysis of the p18 sequence revealed a region spanning 105 residues of the carboxy-terminal two-thirds of p18 with significant protein sequence similarity (32% identity over a 107-amino-acid region) to a highly conserved domain in the members of the Notch family,

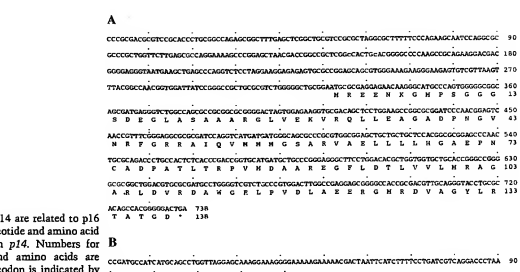


Figure 2. p18 and p14 are related to p16 and Notch. (*A*) Nucleotide and amino acid sequences of human *p14*. Numbers for both nucleotides and amino acids are given, and the stop codon is indicated by an asterisk (*). (*B*) Nucleotide and amino acid sequences of human *p18*. Numbers for both nucleotides and amino acids are given, and the stop codon is indicated by an asterisk. (*C*) Sequence comparison of p14, p16, and p18. The entire coding regions of p18 [168 residues], p14 [138 residues], and p16 [148 residues] were aligned, and only identical amino acid residues between the three sequences are indicated. Two single-residue gaps were introduced as represented by hyphens. (*D*) p18 is related to Notch proteins. Shown is a comparison of 105 amino acid residues of the p18 sequence with four members of the *Notch* gene family. Only residues that are identical to all five sequences are in boldface type. Two single-residue gaps, represented by hyphens, were introduced in the p18 sequence. The number preceding each sequence indicates the position of the first amino acid residue in each gene. (*TAN1-Hs*) The translocation-associated *Notch* homolog of humans [Ellisen et al. 1991]; (*Xotch-X1*)*a Xenopus* homolog of the *Drosophila Notch* gene (Coffman et al. 1990), (*Notch-Br*) a zebrafish homolog of the *Drosophila Notch* gene (Bierkamp and Campos-Ortega 1993); (*Notch-Dm*) the *Drosophila Notch* gene (Wharton et al. 1985).

which function in the developmental determination of embryonic cell fates (Fig. 2D, for review, see Greenwald and Rubin 1992). p18 exhibited greater sequence identity to the human Notch homolog, TAN1 (37%). Within this region, the shorter carboxy-terminal ends of p16 (91 amino acids) and p14 (70 amino acid) also share considerably lower, but potentially significant, sequence similarities to the *Notch* genes (21% and 24% identity to TAN1, respectively). This region in p18, p16, and p14 and members of the Notch family of proteins shows a limited sequence similarity to the previously identified cdc10/SWI6 ankyrin repeat (Breeden nd Nasmyth 1987; Andrews and Herskowitz 1989; Ellisen et al. 1991; Serrano et al. 1993). Furthermore, the amino-terminal region of p18 (residues 60–123) also contains significant sequence similarity (47%) to a yeast phosphate-regulated PHO80–PHO85 CDK inhibitor PHO8 [residues 561–646 (Coche et al. 1990; Schneider et al. 1994)]. As this region of p18 shows considerably higher similarity to the members of the Notch family than to any other ankyrin re-

peat-containing proteins, it is not clear whether this region in p18 and Notch represents a distinct subset of ankyrin sequences or defines an as-yet-uncharacterized functional relationship shared by p18 and Notch.

p18 specifically interacts with CDK6 and CDK4

We assessed the specificity of the interaction of p18 with known CDKs in two ways. First, yeast cells were cotransformed with a plasmid encoding a Gal4 activation domain–p18 fusion protein (Gal4^{ad}–p18) and a plasmid encoding a Gal4-DNA-binding domain–CDK fusion (Gal4^{bd}–CDK). Transformed cells were streaked on plates with or without histidine. The Gal4^{ad}–p18 strongly interacted with the Gal4^{bd}–CDK6 fusion and very weakly with Gal4^{bd}–CDK4 but not with Gal4^{bd}–CDK3 nor with Gal4^{bd}–CDK2 (Fig. 3A). Second, the specificity of this interaction was studied in a cell-free system. A fusion protein consisting of glutathione *S*-transferase and p18 (GST–p18) was expressed in bacteria and purified GST–p18 was mixed with equivalent amounts of the known CDK proteins that had been in vitro translated with [^{35}S]methionine labeling. The GST–p18 fusion was recovered from the different mixtures on glutathione–agarose beads, and proteins bound to GST–p18 were resolved by SDS-PAGE. Consistently, GST–p18 bound strongly to CDK6, weakly to CDK4, and did not bind to any other CDKs (Fig. 3B).

In vivo association of p18 with CDK6 and CDK4

To study the interaction of p18 with other cellular proteins in vivo, rabbit antisera were raised against the entire p18 protein (see Materials and methods). This antibody reacts strongly with human p18 protein as tested by immunoblotting with purified p18 protein and by immunoprecipitation with in vitro-translated p18 protein. The antibody also cross-reacts very weakly with human p16 protein in immunoprecipitation but not with any of the CDKs that we have tested (data not shown for CDC2–CDK5). An acute lymphoblastic leukemia cell line, CEM, was chosen for the analysis of p18 protein interaction in vivo because it has a homozygous deletion of the *p16* locus and expresses *p18* mRNA at a high level (data not shown). [^{35}S]Methionine-labeled lysates were prepared from CEM cells and immunoprecipitated with the anti-p18 antibody in parallel with antibodies specific to CDK4 and CDK6 (Fig. 1B). The anti-p18 antibody precipitated several cellular proteins that were not seen in the corresponding precipitation with preimmune serum from the rabbit used to generate the anti-p18 antibody. An 18-kD protein that comigrated with in vitro-translated p18 protein (data not shown) was later confirmed as p18 by immunoblotting (Fig. 4A). The anti-p18 antibody also coprecipitated two polypeptides that correspond to CDK4 and CDK6, respectively, as determined by gel mobility (Fig. 1B) and immunoblotting (Fig. 4A). Reciprocally, anti-CDK6 antibody coprecipitates an 18-kD protein with CDK6, which comigrates with the 18-kD protein present in the anti-p18 immunocomplex and is effectively competed by the preincubation of the antibody with an antigen peptide (Fig. 1B, lanes 5, 6).

In addition to the 18-kD band, both anti-p18 and anti-CDK6 antisera precipitate a 20-kD polypeptide (p20, Fig. 1B). Partial V8 peptide mapping of p20 gave rise to a different pattern from both p18 and p16 (data not shown).

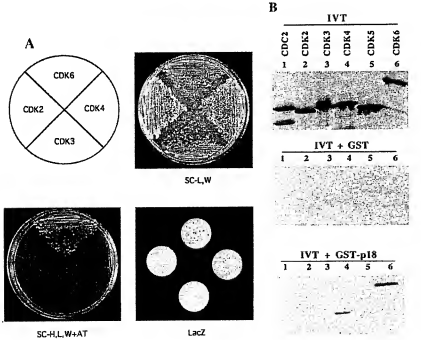


Figure 3. Interaction of p18 with CDKs. (*A*) Yeast cells were simultaneously transformed with a plasmid expressing a Gal4^{bd}–p18 fusion and with plasmids expressing Gal4^{ad} fused to the indicated CDKs. Cells were streaked on nonselective medium with histidine (SC-L W) and selective medium without histidine/with 40 mM 3-amino-1, 2, 3-triazole (SC-H, L,W + AT). Staining of LacZ expression is shown at *bottom right*. (*B*) Equal amounts of purified GST or GST–p18 fusion protein were incubated with equal amounts of each of the six [^{35}S]methionine-labeled, in vitro-translated CDK proteins and recovered from the different mixtures on glutathione–agarose beads. Proteins bound to GST or GST–p18 were resolved by SDS-PAGE.

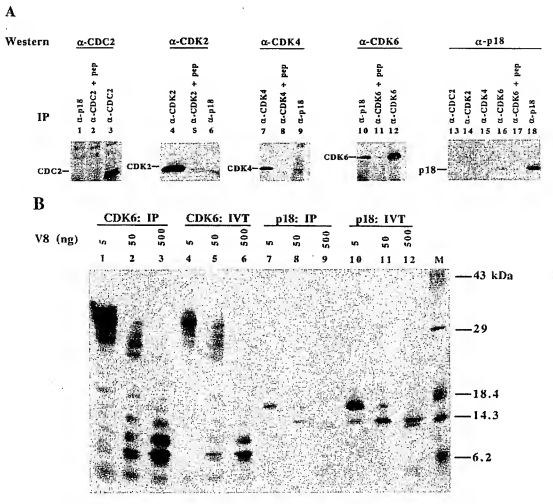


Figure 4. In vivo association of p18 with CDK4 and CDK6. (*A*) Coupled immunoprecipitation and immunoblotting. CEM cell lysates were immunoprecipitated with antibodies as indicated. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with five different antibodies as indicated at the *top*. (*B*) Comparison of V8 proteolysis patterns of immunoprecipitated [lanes 1–3] and in vitro-translated CDK6 [lanes 4–6]; p18 co-immunoprecipitated by anti-CDK6 antibody [lanes 7–9] and in vitro-translated p18 [lanes 10–12].

This band was effectively competed by a CDK6 antigen peptide in anti-CDK6 immunoprecipitates and was not seen in immunoprecipitation with preimmune serum, suggesting that it may represent a p18-related, CDK6-associated protein.

Coupled immunoprecipitation–Western blotting experiments were carried out to study the interaction of p18 with known CDK proteins. CEM cell lysates were immunoprecipitated with the anti-p18 antibody, as well as with a battery of antibodies specific to four different CDKs, and were probed in Western blotting with either anti-CDK or anti-p18 antibodies (Fig. 4A). Consistent with the results obtained from coupled [^{35}S]-IP experiments (Fig. 1B), the anti-p18 antibody quantitatively precipitated p18 (Fig. 4A, lane 18) and also precipitated CDK6 (lane 10), and precipitated CDK4 more weakly (lane 9) but not CDC2 or CDK2. Reciprocally, anti-CDK6 antibody also immunoprecipitated p18 that was effectively competed by the antigen peptide (lanes 16 and 17). Failure to detect p18 in anti-CDK4 precipitates (lane 15) perhaps reflects the weak interaction between the two proteins and the low abundance of CDK4 in CEM cells.

Finally, to confirm the identity of the 18-kD band present in the anti-CDK6 immunoprecipitate as well as CDK6 itself, both bands were excised from an SDS–polyacrylamide gel, partially digested with V8 protease, electrophoretically separated, and compared with similarly V8-digested in vitro-translated p18 and CDK6 (Fig. 4B). The proteolysis pattern of the 18-kD protein derived from anti-CDK6 immunoprecipitation is identical to that of in vitro-translated p18 (note the two doublets in Fig. 4B, lanes 9,12). The p18 pattern is different from that of CDK6 (Fig. 4B) and from those of p14, p15, and p16

precipitated by the same anti-CDK6 antibody in HaCaT cells (data not shown), confirming that the 18-kD band precipitated by the anti-CDK6 antibody in CEM cells corresponds to p18. Interestingly, preferential expression of p18 was observed in a variety of hematopoietic cells (data not shown) which, together with its strong interaction with CDK6, correlates with the previously described preferential expression of CDK6 protein in these cells (Meyerson and Harlow 1994; Tam et al. 1994).

p18 inhibits CDK6 kinase activity

To test directly whether p18 inhibits the kinase activity of CDK6 with which it interacts strongly both in vitro and in vivo, we assayed for its inhibition of cyclin D2 and CDK6 expressed in insect Sf9 cells. Human p18 protein was expressed in bacteria and purified to near homogeneity. Increasing amounts of purified p18 protein were incubated with equal amounts of Sf9 cell lysates prepared from insect cells infected with baculoviruses expressing both CDK6 and cyclin D2. p18 inhibits cyclin D2–CDK6 activity in a dose-dependent manner (Fig. 5A) but had no effect on the kinase activity of CDK2 (Fig. 5B), CDC2, and cyclin A enzymes (data not shown). We have not tested the inhibitory activity of p18 on CDK4. Given its weak interaction, we predict that p18 will also inhibit the activity of CDK4 but with less potency.

Expression of p18 *mRNA*

Northern blot analysis was carried out to determine the expression of *p18* mRNA in different human tissues. These analyses were performed under high-stringency conditions for hybridization with the *p18* cDNA probe, and they revealed several discrete bands (Fig. 6). The relative intensity of these bands appears to vary in different tissues (e.g., cf. lanes 2, 6, and 8). At least two of these bands may result from different transcription initiations, because we have isolated from a cDNA library two classes of *p18* cDNA clones that differ in their 5′ ends. Sequence analysis revealed a class of two cDNA clones with an extension of ~500 nucleotides 5′ to the *p18* sequence presented in Figure 2B, but it does not alter the coding capacity of *p18* (data not shown). Nevertheless, we cannot exclude the possibility that one of these discrete bands on the Northern blot may correspond to a yet-unidentified gene related to *p18*. Whether the differential transcription initiation of *p18* may play a role in the regulation of *p18* function in vivo is not clear.

The level of *p18* mRNA varies dramatically between different human tissues (Fig. 6). The highest level was observed in human skeletal muscle, and moderate levels were present in pancreas and heart. At the other end of the spectrum, *p18* mRNA was almost undetectable in placenta. Consistently, a drastic variation in the level of *p18* mRNA was also seen in different cell types (Y. Li and Y. Xiong, unpubl.). These results demonstrate a very different expression pattern for *p18* mRNA than for two other cyclin–CDK kinase inhibitors, p27 and p21, that appear to be expressed in most human tissues at a similar level (Fig. 6; Polyak et al. 1994b) indicating a tissue-specific regulation of *p18* gene expression and the possible involvement of *p18* function in cellular differentiation and development.

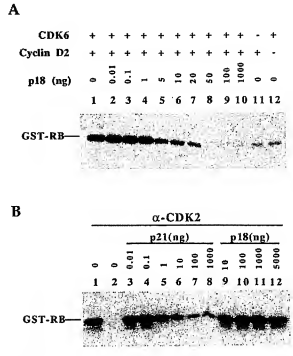


Figure 5. p18 inhibits the activity of cyclin D2–CDK6 kinase. (*A*) Increasing amounts (in nanograms) of purified p18 protein were added to a 25-μl kinase reaction containing the same amount of lysate derived from Sf9 insect cells that had been infected with baculovirus-expressing cyclin D2 and CDK6 proteins as indicated. Phosphorylation of GST–Rb-carboxy-terminal fusion protein was analyzed by SDS-PAGE followed by autoradiography. (*B*) Increasing amounts (in nanograms) of purified p18 or p21 protein were added to the same amount of CEM cell lysate prior to immunoprecipitation by an anti-CDK2 antibody. Immunoprecipitated CDK2 kinase activity was assayed with the same GST–Rb–carboxy-terminal fusion protein as substrate, and phosphorylation of the GST–Rb-carboxy-terminal was analyzed by SDS-PAGE followed by autoradiography. (Lane 2) Immunoprecipitation with anti-CDK2 antibody that has been preincubated with an excess amount of a competing CDK2 peptide antigen.

Chromosomal localization and genomic structure of p18

Using the cDNA fragments as probes, we have isolated genomic fragments containing *p14*, *p16*, and *p18* from a human placenta library and have partially determined their genomic structure. DNA sequencing and comparison with cDNA sequences revealed that the coding regions of all three genes were interrupted by an intron at the same position, corresponding to nucleotide position 477 in *p14* (Fig. 2A), nucleotide 144 in *p16* (Serrano et al.

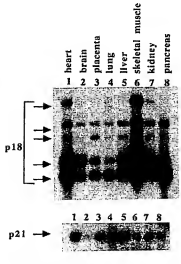


Figure 6. Expression of *p18* mRNA. Expression of *p18* mRNA in different human tissues. Poly(A)$^+$ RNAs (2 μg) from eight different human tissues, as indicated at the *top*, were hybridized with a 1.6-kb probe derived from *p18* cDNA. Five discrete bands were detected by this probe and are indicated by arrows.

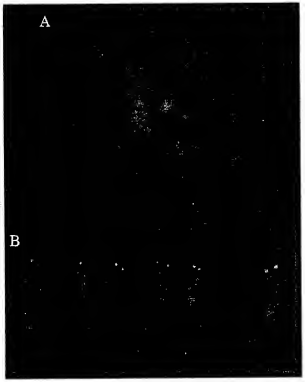
1993), and nucleotide 222 in *p18* (Fig. 2B). These results demonstrate that *p18*, *p16* (*INK4/MTS1*), and *p14* (*MTS2/INK4B*) have evolved from a common ancestor; thus, they constitute a family of evolutionarily related genes.

To determine the chromosomal location of *p18*, we used fluorescence in situ hybridization (FISH). The authenticity of the probe used in the hybridization was verified by DNA sequencing (Fig. 7D). Digital imaging microscopy revealed exclusive hybridization to human chromosome 1p32 (Fig. 7A–C). Abnormalities in the distal arm of chromosome 1 (1p32-pter), and in 1p32 specifically, have been reported in a variety of human tumors, including breast cancer (Hainsworth et al. 1991; Bieche et al. 1994), pancreatic cancer (Bardi et al. 1993), melanoma (Bale et al. 1989; Sokova et al. 1992), leiomyosarcoma (Sreekantaiah et al. 1993), and neuroblastoma (Schleiermacher et al. 1994). The biochemical and biological properties of p18, inhibition of CDK activity, and suppression of cell growth (see below), both suggest a potential tumor suppression function for p18. Considering the recent discoveries that *p16*, a homolog of *p18*, is deleted or mutated at a high frequency in a wide variety of human tumor-derived cell lines (Kamb et al. 1994a; Nobori et al. 1994) and also in several specific types of primary tumors (Caldas et al. 1994; Hussussian et al. 1994; Kamb et al. 1994b; Mori et al. 1994; Spruck et al. 1994), this possibility is clearly worth exploration.

Cell growth suppression by p18

The biochemical analysis of p18 has demonstrated that it acts as an inhibitor of CDK6 (most likely CDK4 as well). As such, one would predict that overexpression of p18 in vivo may inhibit cell proliferation and growth — a property that has been shown previously for two other cyclin–CDK inhibitors, p21 (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993a; Noda et al. 1994) and p27 (Toyoshima and Hunter 1994; Polyak et al. 1994b). We employed a colony formation assay to directly test this prediction (Zhu et al. 1993). The full-length coding region of *p18* was placed under the control of a strong promoter of cytomegalovirus (CMV) that also carries a neomycin-resistance (*neo*) gene, and the resultant plasmid, pCMV–p18, was transfected into human U-2 OS osteosarcoma cells. The biological effect of ectopic expression of *p18* was measured by the scoring of the number of G418-resistant colonies 3 weeks after the transfected cells were cultured in a medium supplemented with G418 and by comparison with the number of G418-resistant colonies obtained from a parallel transfection with the parental vector plasmid pcDNA3 or a plasmid expressing antisense *p18* (pCMV–p18AS; see Materials and methods). Introduction of full-length *p18* into U-2 OS cells significantly reduced their ability to grow as G418-resistant colonies (Table 1). Essentially the same result was obtained for *p16* (Table 1). These results provide evidence to support a function of *p18* (and *p16*) in negative regulation of cell growth, a notion that is consistent with its biochemical property of inhibiting the activity of cyclin-dependent kinases.

The function of pRb is known to be down-regulated by cell cycle-dependent phosphorylation and D-type cyclins and their associated kinases, primarily CDK4 and CDK6, have been strongly implicated as physiological pRb kinases. The substrate specificity of CDK4 and CDK6 kinases and the specific interaction of p18 with CDK6 and CDK4 provoked us to test whether cell-growth suppression by *p18* is dependent on the existence of endogenous pRb. To test this possibility, another line of human osteosarcoma cells, Saos-2, was transfected with each of the four plasmid DNAs in parallel with the U-2 OS cells as described above. U-2 OS and Saos-2 cells express a similar level of CDK4 and CDK6 proteins as determined by immunoprecipitation (data not shown). Saos-2 cells express no endogenous wild-type pRb because of a deletion in exons 21–27 of the *RB1* gene (Shew et al. 1990; our confirmatory results). Strikingly, expression of the same pCMV–p18 and pCMV–p16 DNA that inhibited growth of U-2 OS cells had no apparent effect on the growth of Saos-2 cells, as measured by the scoring of the number of G418-resistant colonies and by comparison with parallel transfections with the vector pcDNA3 plasmid or plasmids expressing antisense *p18* and antisense *p16* (pCMV–p18AS and pCMV–p16AS), respectively. High-level expression of p16 and p18 proteins and increased association with CDK4 and CDK6 in these stable transformants were verified by immunoprecipitations (data not shown). These results are in contrast to the previously reported growth suppression by p21 and p27, which inhibited the growth of Saos-2 as well as that of a number of additional tumor cell lines (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993a; Noda et al. 1994; Toyoshima and Hunter 1994). These are the first



D

```
                N G F G R T A L Q                    V X K L G X P
p18: cDNA       AATGGATTTGGAAGGACTGCGCTGCA3          GTCACGAAACTTGGAAATCCC
p18: Genomic    AACGGATTTGGAAGGACTGCGCTGCAGGTGG....TTTTCCAGGTCACGAAACTTGGAAATCCC

                N S Y G R R P I Q                    V X X X G S A
p16: cDNA       AATAGTTACGGTCGGAGGCCGATCCAG          GTCATGATGATGGGCAGCGCC
p16: Genomic    AATAGTTACGGTCGGAGGCCGATCCAGGTGGG....TCTGGCAGGTCATGATGATGGGCAGCGCC

                N R F G R R A I Q                    V X X X G S A
p14:cDNA        AACCGTTTCGGGAGGCGCGCGATCCA3          GTCACGATGATGGGCAGCGCC
p14:Genomic     AACCGTTTCGGGAGGCGCGCGATCCAGGTCTG....TCTGGCAGGTCATGATGATGGGCAGCGCC
```

Figure 7. Chromosomal location and genomic structure of *p18*. (*A*) A biotin-labeled *p18* genomic clone maps to human chromosome 1 (green). The C/Q banding pattern was generated with the DNA counterstain DAPI (blue). (*B*) Six different chromosomes 1, at a higher magnification, showing hybridization of *p18* to 1p32. (*C*) An ideogram of chromosome 1. The bar (*right*) represents the range of *p18* signals. (*D*) An intron interrupts *p14*, *p16*, and *p18* at the same position. Partial genomic structures for *p14*, *p16*, and *p18* were determined by DNA sequencing and comparison with their cDNA sequences. Introns interrupt the coding regions of *p14* at a position corresponding to nucleotide 477 (Fig. 2A), *p16* at a position corresponding to nucleotide 144 (Serrano et al. 1993), and *p18* at a position 222 (Fig. 2B).

examples of results showing that growth suppression by a cyclin–CDK inhibitor is cell-line specific and may be pRb dependent. They provide further in vivo support for the conclusion that p16 and p18 only interact specifically with CDK4 and CDK6 but not with other CDKs because no effect was observed when they were overexpressed in pRb-deficient Saos-2 cells.

Discussion

In mammalian cells the number of small cell-cycle regulatory proteins identified by virtue of their ability to interact physically with cyclin or CDK proteins is increasing rapidly. The sequence similarity between two of these previously identified CDK-associated proteins, p21 and p27, defines a family of CDK inhibitors (Polyak et al. 1994a; Toyoshima and Hunter 1994). In this report we characterized two novel genes encoding CDK4- and CDK6-specific inhibitors, *p18* and *p14*. The sequence, functional, and evolutionary similarities among p18, p16, and p14 define a second family of CDK inhibitors that were evolved from a common ancestor. Our in vivo analysis of CDK4- and CDK6-associated proteins revealed at least two additional proteins, p15 and p20, that share functional or antigenic similarity with p16 and p18, further indicating that a potentially large number of small cyclin- and/or CDK-associated proteins, yet to be identified, are present in cells.

p14, p16, and p18 do not share any detectable sequence similarity with p21 and p27. In addition, the two families of CDK inhibitors exhibit two substantially different properties. First, with differing affinities, both p21 and p27 (p21 in particular) can interact with and inhibit the activity of a wide range of CDKs (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993a; Zhang et al. 1993; Polyak et al. 1994a; Slingerland et al. 1994; Toyoshima and Hunter 1994), whereas p14, p16, and p18 (potentially p15 and p20 as well) only interact with the D-type CDKs, CDK4 and CDK6 (Figs. 1,3, and 4; Serrano et al. 1993; Xiong et al. 1993b; Hannon and Beach 1994). CDK4 and CDK6 are related most closely to each other and form a distinct sub-branch within the CDK family (Meyerson et

Table 1. *Inhibition of cell growth by* p18 *correlates with pRb status*

Recipient cells	transfected DNA	Growth suppression by *p18* and *p16*: average number of G418-resistant colonies (experiment number) 1	2	3
U-2 OS (Rb+/+)	pCMV–p18	3	2	11
	pCMV–p18AS	41	68	62
	pCMV–p16	2	5	9
	pCMV–p16AS	45	36	44
	pcDNA3	N.D.	N.D.	54
Saos-2 (Rb−/−)	pCMV–p18	86	N.D.	71
	pCMV–p18AS	82	N.D.	74
	pCMV–p16	80	45	67
	pCMV–p16AS	83	39	74
	pcDNA3	N.D.	N.D.	69

A colony formation assay was employed to test growth suppression by *p18*. The full-length coding region of *p18*, as well as that of *p16*, was placed under the control of a strong promoter of CMV that also carries a *neo* gene in both the sense and antisense orientations, and the resultant plasmids, pCMV–p18, pCMV–p18AS, pCMV–p16, and pCMV–p16AS, were transfected into human U-2 OS and Saos-2 osteosarcoma cells. Although an apparently normal $p105^{pRb}$ protein was readily detectable in U-2 OS cells, it was not detected in Saos-2 cells—presumably because of a deletion in exons 21–27 of the *RB1* gene (Shew et al. 1990). The biological effect of ectopic expression of *p18* and *p16* was measured by scoring of the number of G418-resistant colonies after 3 weeks of culturing of the transfected cells in a media supplemented with G418, and comparison with the number of G418-resistant colonies obtained from a parallel transfection with parental vector plasmid (pcDNA3) or a plasmid expressing antisense *p18* (pCMV–p18AS) and *p16* (pCMV–p16AS). [N.D.] Not determined.

al. 1992). Second, whereas p21 (Harper et al. 1993; Xiong et al. 1993a; Zhang et al. 1994) and p27 (Firpo et al. 1994; Hengst et al. 1994; Polyak et al. 1994a) inhibit the activity of a CDK by forming a ternary complex that may actually stabilize the association of the regulatory cyclin and the catalytic CDK, p14, p16, and p18 were only detected in association with CDKs (4 and 6), but not with cyclins, and, conversely, no cyclin protein was seen in p16 and p18 complexes (Fig. 1; Serrano et al. 1993; Xiong et al. 1993b; Hannon and Beach 1994). These observations suggest that p14, p16, and p18 may inhibit the kinase activities of CDK4 and CDK6 by forming a binary complex with the CDK catalytic subunit and disrupting the cyclin D–CDK association (Fig. 8A). Identification of these two families of CDK inhibitors raises the intriguing possibility that additional families may exist that bind preferentially to other catalytic CDK subunits, or that can form a binary complex with the regulatory cyclin subunit (e.g., cyclin A-associated p19; Xiong et al. 1993b), disrupting the cyclin–CDK association.

The growth-inhibitory function of pRb is down-regulated by cell cycle-dependent phosphorylations that are likely to be catalyzed by cyclin-dependent kinases (for a review, see Ewen 1994). D-type cyclins and their associated kinases (primarily CDK4 and CDK6) have been strongly implicated as physiological pRb kinases (Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993; Meyerson and Harlow 1994). As expected from their common biochemical properties, inhibition of the kinase activity of CDK4 and CDK6 (Fig. 5; Serrano et al. 1993) and overexpression of *p18* and *p16* significantly reduced cell growth as measured by a colony formation assay (Table 1). It remains to be determined whether growth suppression by p18 and p16 is attributable to cell death or cell-cycle arrest. Strikingly, growth suppression by the ectopic expression of p18 and p16 is cell-line specific. Although the overexpression of p18 and p16 failed to

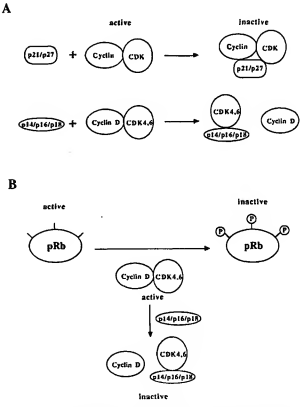


Figure 8. Mechanisms for CDK inhibition and growth suppression by p14, p16, and p18. (*A*) Two families of cyclin–CDK inhibitors. p21 and p27 form ternary complexes with and inhibit the activity of most, if not all, cyclin–CDK enzymes. p14, p16, or p18 binds to the catalytic subunit CDK4 or CDK6 to form a binary complex and may dissociate the activating D-type cyclin, thereby inactivating the kinase. (*B*) Dependence on pRb for growth suppression by p14, p16, and p18. Hypophosphorylated pRb actively suppresses cell-cycle progression. The growth-suppressing activity of pRb may be inactivated in two ways: by mutation (as in Saos-2 cells) or by hyperphosphorylation. Inactivation of CDK4 and CDK6 by p14, p16, or p18 prevents hyperphosphorylation of pRb, maintaining the active growth-suppressing state.

produce appreciable numbers of G418-resistant colonies in U-2 OS cells, it had no detectable effect on the growth of Saos-2 cells (Table 1). Using HeLa cells that do not contain functional pRb as a result of the expression of type 18 papilloma viral E7 proteins, we obtained essentially the same results—that no growth suppression was observed in transfections with either p18 or p16 overexpression plasmids (Y. Li and Y. Xiong, unpubl.). Although other differences between these cell lines could also contribute to the observed differences in growth suppression by p18 and p16, our results can be explained most simply at this time by the difference in their pRb status. Although a normal $p105^{pRb}$ protein was readily detectable in U-2 OS cells, it was not detected in Saos-2 cells—apparently because of a deletion in exons 21–27 of the *RB1* gene (Shew et al. 1990). Lacking functional pRb, and therefore being devoid of a functional target, overexpression of p16 or p18 and the resulting inhibition of CDK4 and CDK6 kinase activity is futile in Saos-2 cells (Table 1; Fig. 8B). This model is supported by the observation that high levels of p16 and p18 are expressed and tolerated in cells lacking functional pRb and that p16 and p18 only interact with and inhibit the activity of CDK4 and CDK6 (Figs. 3 and 4; Xiong et al. 1993b; Serrano et al. 1993; Li et al. 1994). The model is entirely consistent with the observations that DNA tumor virus oncoproteins or pRb mutations can relieve the requirement of cells for cyclin D1 function in G_1 (Lukas et al. 1994; Tam et al. 1994). It also suggests that pRb proteins are the critical, if not exclusive, in vivo targets of CDK4 and CDK6.

In summary, we have identified four new CDK4- and CDK6-associated small cellular proteins and isolated the gene encoding two of these proteins, p18 and p14. p18 interacts specifically with and inhibits the activity of CDK6. Ectopic expression of p18 and p16 suppresses cell growth in a manner that appears to correlate with the existence of an endogenous wild-type pRb function. These results provide evidence in support of the hypothesis that CDK4 and CDK6 are physiological pRb kinases and suggest a potential mechanism by which members of the *p14/p16/p18* family inhibit cell growth (Fig. 8B), that is, inhibiting the activity of CDK6 and CDK4 kinases, thereby preventing the phosphorylation of pRb and keeping pRb in its active growth-suppressing state.

Materials and methods

Cell lines and cell culture

All cells were cultured in a 37°C incubator with 5% CO_2. HaCaT is a cell line of spontaneously immortalized human keratinocytes (Boukamp et al. 1988). These cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). CEM is a human acute lymphoblastic leukemia cell line (ATCC CCL 119), which was cultured in RPMI-1640 supplemented with 10% FBS.

Yeast two-hybrid screen

For two-hybrid screening, the complete open reading frame of human CDK6 was inserted into pGBT8, a modified form of pGBT9 (Bartel et al. 1993). This vector directs the expression of a fusion between the DNA-binding domain [amino acids 1–147] of Gal4 and the entire CDK6 protein through a crippled ADH promoter as confirmed by Western blotting with an anti-CDK6 antibody. pGBT–CDK6 plasmid DNA was cotransformed with a human HeLa cDNA library constructed in the vector pGADGH (*LEU2* marker, Clontech, Inc., Palo Alto, CA] into Y190 yeast cells [*MATa, gal4, gal80, his3, trp1-901, ade2-101, ura3-52, leu2-3,112, URA3::GAL–lacZ, LYS2::GAL–HIS3*] by lithium acetate methods. Transformants were plated on yeast dropout medium lacking histidine, tryptophan, and leucine and containing 40 mM 3-amino-1,2,4, triazole (3-AT). We estimated that $\sim 5 \times 10^6$ transformants were screened. His^+ colonies were purified and tested for *lacZ* expression by β-galactosidase staining. After retransformation into yeast cells to confirm their specific interaction with CDK6, the library plasmids were recovered and analyzed by sequencing.

Nucleic acid procedures

Most molecular biology techniques were essentially the same as described by Sambrook et al. (1989). Phagemid vectors pUC118, pUC119, or pBlueScript were used as cloning vectors. DNA sequences were determined either by a chain-termination method by use of a Sequenase kit [U.S. Biochemical, Cleveland, OH], or on an Automated Sequencing System [373A, Applied Biosystems].

To isolate *p14/MTS2* cDNA clones, two oligonucleotide primers, a 5′ primer (5′-AGGATCCATGGTGATGATGGG-CAGCGCCCGC-3′) and a 3′ primer (5′-GAAGCTTGGGTAA-GAAAATAAAGTCGTTG-3′), specific to *MTS2* were designed on the basis of the previously published *MTS2* genomic sequence (Kamb et al. 1994a) and used in PCR amplification with template prepared from a human HeLa cDNA library (Stratagene, La Jolla, CA). A 319-bp fragment was amplified by these two primers, purified, and used as a probe to screen the same HeLa cDNA library for the full-length cDNA of *p14*.

To isolate a full-length cDNA of *p18*, a HeLa cDNA library was screened with a probe derived from plasmid 6H10 obtained from two-hybrid screening. To isolate genomic clones containing the *p18* gene, the same 1.6-kb DNA fragment containing *p18* cDNA derived from clone H19 was used as a probe to screen a human placenta genomic library cloned in λ FIX II (Stratagene). Four positives were obtained and one clone, λp18-G1, was characterized further by subcloning and nucleotide sequencing. After confirmation of its identity by sequencing, λp18-G1 was used as a probe for the chromosomal location of *p18* by the FISH technique.

Poly[A]$^+$ RNA [2 μg] was isolated from different human tissues, resolved on a 1.2% agarose gel, and transferred to a nylon membrane (Clontech). A 1.6-kb DNA fragment containing the p18-coding region, as well as 3′-untranslated sequences, was labeled by the random priming method and used as a probe for Northern hybridization.

Chromosomal localization

Phage DNA [map2 μg], bearing an ~16-kb *p18* insert, was nick translated with biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN] and hybridized to human metaphase chromosomes as described [Lichter et al. 1990]. Human Cot 1 DNA (GIBCO BRL, Gaithersburg, MD] was used as a repetitive sequence competitor. After hybridization overnight, the slides were washed in 50% formamide/2× SSC at 37°C, followed by washes in 1× SSC at 60°C. After detection with FITC-conjugated avidin (Vector Laboratories, Burlingame, CA], the DNA counterstain DAPI was used to generate a G/Q-banding pattern on the chromo-

somes. Twenty-one metaphases were imaged by use of a cooled charge-coupled device camera (Photometrics), revealing exclusive hybridization to chromosome 1. The 16-bit source images were saved as normalized 8-bit gray scale data files by use of CCD image capture (Yale University, New Haven, CT.). Proper registration of the source images was accomplished in two ways: by use of highly plane parallel bandpass filters (Ballard and Ward 1993), and with an excitation filter slider (CRG Electronics), which selectively excites individual fluorochromes that are viewed with a triple-bandpass emission filter (ChromaTech). Equivalent results were obtained with both methods. Merging and 24-bit pseudocoloring were accomplished by use of Gene Join (Yale University) on an Apple Macintosh computer; color prints were produced by use of Adobe Photoshop 2.5.1 (Adobe Systems) and a dye sublimation printer (Kodak). The cytogenetic location in band 1p32, determined by use of DAPI banding, was confirmed by the averaging of FLpter measurements from five metaphase spreads [range 0.17–0.22; Lichter et al. 1990].

Antibodies and immunochemistry procedures

To produce anti-p18 antibody, a 0.5-kb DNA fragment containing the full-length coding region (168 amino acid residues) of human *p18* was generated by PCR, subcloned into a T7 expression vector, pET-3d (Studier et al. 1990), and introduced into the *Escherichia coli* strain BL21(DE3). Subsequent procedures for the expression and purification of p18 proteins and the production of rabbit polyclonal anti-p18 antibody were as described previously for the production of anti-cyclin D1 antibody (Xiong et al. 1991, 1992).

With the exception of CDK6, all cyclin and CDK antibodies and the procedures for [^{35}S]methionine metabolic labeling, immunoprecipitation, and immunoblotting have been described previously (Xiong et al. 1993b). Anti-CDK6 peptide antibodies used in this study were described and provided by Meyerson and Harlow (1994).

Kinase assay

To assay for inhibitory activity, p18 protein was expressed in *E. coli*, as described above, and purified to near homogeneity (>95% purity; a detailed procedure for the p18 protein purification will be described elsewhere; H. Ke and Y. Xiong, in prep.). To prepare substrates for cyclin–CDKs, a DNA fragment containing the carboxy-terminal 137 amino acid residues of pRB was generated by PCR and subcloned into pGEX-KG (Guan and Dixon 1991). The resultant GST–RB-carboxy-terminal fusion protein was purified and used as a substrate in the kinase activity assay as described previously (Meyerson and Harlow 1994). To assay for the inhibitory activity of p18 on CDK6–cyclin D2 (Fig. 5A), insect Sf9 cell lysates containing CDK6, cyclin D2, or their combination were prepared as described previously (Kato et al. 1993; Meyerson and Harlow 1994) and provided by Harper (Harper et al. 1993). Sf9 cell lysate (2 μl) containing CDK6 and cyclin D2 was mixed with 1 μl of purified p18 protein diluted with extraction buffer (50 mM Tris-HCl at pH 7.5, 50 mM NaCl, 1 mM EDTA, 10% glycerol) for 15 min at room temperature. Twenty microliters of kinase assay buffer (50 mM HEPES at pH 7.3, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) was then added to the mixture and incubated further at room temperature for an additional 5 min. To assay for inhibitory activity on other cyclin and CDK enzymes (Fig. 5B), 1 μl of p18 or p21 protein diluted with extraction buffer was incubated with an equal amount of CEM cell lysate (NP-40 lysis buffer; Xiong et al. 1993b) for 30 min at 4°C prior to immunoprecipitation with antibodies specific to CDC2, CDK2, and cyclin A. Immunoprecipitates were washed twice with lysis buffer and twice with kinase assay buffer. Protein A–agarose beads were resuspended in a final volume of 30 μl of kinase assay buffer. The kinase reaction was started by addition of 1 μg of purified GST–RB–carboxy-terminal protein, 5 μM cold ATP, and 10 μCi of [γ-^{32}P]ATP and incubation for 30 min at 30°C. At the end of the reaction, 20 μl of 2× SDS sample buffer was added, and the phosphorylated proteins were electrophoresed on 15% denaturing polyacrylamide gels. The gels were stained with Coomassie blue prior to autoradiography.

In vitro translation and in vitro-binding assay

[^{35}S]Methionine-labeled CDKs were prepared in vitro with T7 RNA polymerase by use of a TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Prior to the binding assay, the amount of each in vitro-translated protein was normalized after electrophoresis on a 15% denaturing polyacrylamide gel and autoradiography (Fig. 3B, top). A DNA fragment containing the full-length *p18* was generated by PCR and subcloned into the pGEX-KG expression vector for the expression of GST–p18 fusion protein. The fusion protein was purified with GST–agarose beads and washed three times with a large excess volume of binding buffer (the same as that used in immunoprecipitation). Equal amounts of GST or GST–p18 proteins were mixed by rotation with each ^{35}S-labeled in vitro-translated CDK protein for 2 hr at 4°C. After three washes with the binding buffer, bound proteins were released by boiling in SDS sample buffer and electrophoresed on 15% denaturing polyacrylamide gels followed by autoradiography.

Growth suppression assay

To assay for growth suppression by p18 and p16, full-length coding regions of *p18* and *p16*, both sense and antisense orientations, were placed under the control of a strong promoter of an immediate early gene of human CMV in a mammalian expression vector, pcDNA3, that also carries the neomycin resistance gene *neo* (Invitrogen, San Diego, CA). The colony formation assay was carried out according to a previously described procedure (Zhu et al. 1993). Briefly, both U-2 OS and Saos-2 cells were maintained in DMEM supplemented with 10% FBS and antibiotics. The cells were plated 1 day prior to transfection. Two micrograms of each of the resultant four plasmid DNAs, pCMV–p18, pCMV–p18AS, pCMV–p16 and pCMV–p16AS, were transfected with lipid reagents (Lipofectamine Reagent, GIBCO BRL) into the respective cells cultured in six-well dishes (40–60% confluence). Two days after transfection, the cells were split to a 9-cm plate at different dilutions and cultured in the same medium supplemented with 500 μg/ml of G418 (GIBCO BRL). The medium was changed every 4–5 days. Between 2–3 weeks after being cultured in the G418-containing medium, G418-resistant cells were examined under the microscope and colonies were scored and averaged from two or three independent transfections. Only colonies with >50 cells were scored. A considerably higher number of G418-resistant colonies were consistently obtained from transfections with Saos-2 cells than with U-2 OS cells, presumably because of a difference in transfection efficiency between these two cell lines. Consequently, colonies were scored from entire plates of U-2 OS cells and two randomly selected small areas (100 mm^2) for Saos-2 cells.

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Note added in proof

The sequence data for p18 and p14 reported in this paper have been submitted to the GenBank data library under accession numbers U17074 and U17075, respectively.

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